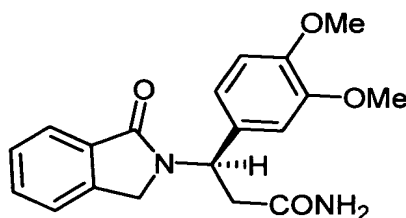


Remarks

Amendment to Claims and Specification

Page 7 of the specification has been amended to correct a typographical error in the structure of (-)-3-(3,4-dimethoxy-phenyl)-3-(1-oxo-1,3-dihydro-isoindol-2-yl)-propionamide. In the structure on page 7 of the specification, "CO" was inadvertently omitted before "NH₂." The correct structure is:



and can be found on pages 37 and 40 of the specification. The typographical error is also apparent for the name of the compound, "(-)-3-(3,4-dimethoxy-phenyl)-3-(1-oxo-1,3-dihydro-isoindol-2-yl)-propionamide," provided immediately above the structure on page 7. No new matter has been added. Appropriate correction is respectfully requested.

Claims 1-16, 20-23, and 33-46 were canceled by preliminary amendment filed on November 17, 2003, without prejudice to Applicants' right to pursue them in one or more continuation, divisional or continuation-in-part applications. Claims 17, 24 and 29-31 have been amended herein to delete the terms of "preventing," "prevention" and "prophylactically" in the present amendments. No new matter has been added by these claim amendments. Accordingly, entry of the foregoing amendments and remarks into the file of the above-identified application is respectfully requested. After the amendments, claims 17-19 and 24-32 are pending in this application.

The Rejection Under 35 U.S.C. § 112 Should Be Withdrawn

On pages 2-8 of the Office Action, claims 17-19 and 24-32 are rejected under 35 U.S.C. § 112, first paragraph, on the ground that the specification is not enabling for a method of treating or preventing a disease ameliorated by the inhibition of PDE4, based on the analysis of factors set forth in *In re Wands*, 858 F.2d 731, 737 (Fed. Cir. 1988) ("*Wands* factors"). Applicants respectfully traverse this rejection.

Although Applicants strongly disagree with the Examiner's allegation that the specification is viewed as lacking enablement for prevention of any diseases recited (page 5,

last paragraph to page 6 of the Office Action), the pending claims have been amended to delete the terms of “preventing” and “prevention,” solely to expedite the prosecution of the present application, and without prejudice to Applicants’ right to pursue them in one or more continuation, divisional or continuation-in-part applications. In view of these amendments and the following discussions, Applicants respectfully submit that the rejection must be withdrawn.

The amended claims recite, *inter alia*, methods of treating a disease or disorder ameliorated by the inhibition of PDE4 in a patient by administering to a patient a therapeutically effective amount of enantiomerically pure (-)-3-(3,4-dimethoxy-phenyl)-3-(1-oxo-1,3-dihydro-isoindol-2-yl)-propionamide, or a pharmaceutically acceptable salt or solvate thereof. That is, the use of a specific compound is claimed, and the claims do not recite unknown compounds.

On page 3, item 3) of the Office Action, the Examiner alleges that there are no disclosures which would enable to identify patients, and predict dosages and routes of administration wherein the compound is to be used for broad indications, that there is no description of any commonality of mechanism of action for the recited drug in the claimed indications, and that none of examples demonstrate the treatment as successful. The Examiner further alleges on page 4, item 4) of the Office Action that no guidance is provided in the specification as to treating or preventing any/all diseases associated with increased PDE4. It is also alleged that the unpredictability in the pharmaceutical arts regarding dosing, patient sensitivities and modes of administration necessitates further support for enabling the treatment or prevention of diseases associated with increased PDE4. (Page 5 of the Office Action). Applicants point out that none of those allegations, alone or in combination, can provide sufficient reason to doubt the fact that the claims are enabled.

The test of enablement is whether one reasonably skilled in the art could make or use the invention from the disclosures in the patent coupled with information known in the art without undue experimentation. *U.S. v. Teletronics, Inc.*, 857 F.2d 778, 785 (Fed. Cir. 1988). The examiner has the initial burden to establish a reasonable basis to question the enablement provided for the claimed invention. *Manual of Patent Examining Procedure* (“MPEP”) § 2164.04 (citing *In re Wright*, 999 F.2d 1557, 1562 (Fed. Cir. 1993)).

Accordingly:

A specification disclosure which contains a teaching of the manner and process of making and using an invention in terms which correspond in scope to

those used in describing and defining the subject matter sought to be patented *must be taken as being in compliance with the enablement requirement ... unless there is a reason to doubt the objective truth of the statements* contained therein which must be relied on for enabling support

* * *

It is incumbent upon the Patent Office, whenever a rejection on this basis is made, to explain why it doubts the truth or accuracy of any statement in a supporting disclosure and to back up assertions of its own with acceptable evidence or reasoning which is inconsistent with the contested statement.

Id. (emphases added).

Further, Applicants respectfully submit that whether the scope of the claim is broad or not is irrelevant to the assessment of the enablement of the claim. The question is whether those skilled in the art would have been able to make and use the claimed invention based on the disclosure. (*See U.S. v. Telectronics, Inc.*, at 785).

Applicants respectfully submit that the pending claims are enabled because the specification “contains a teaching of the manner and process of making and using an invention in terms which correspond in scope to those used in describing and defining the subject matter sought to be patented.” *Id.*

For example, the specification teaches that PDE4 plays a central role in the inflammatory response and that the administration of PDE4 antagonist blocks chronic and acute responses in inflammatory diseases as recited in claim 19. *See, e.g.*, page 2, lines 17-22. Further, the specification discloses that adenosine 3',5'-cyclic monophosphate (cAMP) also plays a role in many diseases and conditions, such as, but not limited to respiratory diseases, asthma and inflammation, and that a primary cellular mechanism for the inactivation of cAMP is the breakdown of cAMP by a family of isoenzymes referred to as cyclic nucleotide phosphodiesterases (PDE). *See, e.g.*, page 3, lines 14-23. The specification and literature references cited in the specification also teach that the inhibition of PDE4 is particularly effective for inhibiting respiratory or inflammatory responses in the diseases. *See, e.g.*, the specification, page 3, lines 14-29. The specification also teaches that the recited diseases are ameliorated by the inhibition of PDE4, and that the diseases can be treated or prevented by the administration of PDE4 inhibitor, which is the compound recited in the pending claims. *See, e.g.*, the specification, page 5, lines 9-24; page 12, line 26 to page 13, line 2; and page 21, lines 11-20. In view of the disclosures, the specification provides

sufficient description on mechanism of action for the recited compound in the claimed diseases.

It is also disclosed that the recited compound can be prepared by synthetic procedures described in Example 2. *See*, the specification, pages 32-40, section 5.2. Dosages and routes of administration of the compound are disclosed, for example, on page 22, line 8 to page 25, line 29 and Examples 7-9 on pages 44-45 of the specification. Therefore, all that is required for those of ordinary skill in the art to practice the claimed invention is to administer the specified amount of the recited compound using the specified routes of administration. In view of the foregoing, the specification provides a sufficient guidance as to treating or preventing the diseases associated with increased PDE4.

Therefore, it is clear that a sufficient guidance is provided in the specification so as to allow those of ordinary skill in the art to make and use the claimed invention, as required by 35 U.S.C. § 112, first paragraph.

Nonetheless, on pages 6-8, items 5)-6) of the Office Action, it is alleged that in the specification there are no working examples, no animal data or extrapolation thereof to the patient recited in the claims, and thus that one skilled in the art would have to undergo an undue amount of experimentation in consideration of factors 1-6 set forth in *In re Wands*. Applicants respectfully disagree with the allegations.

First, Applicants emphasize that the specification does indeed include working examples and animal data. *See*, Examples 1 to 9 on pages 31-45. The specification provides the pharmacokinetic data such as C_{max}, T_{max}, AUC and plasma concentration data taken at 0.5, 1, 2, 4, 7, 10 and 24 hours following the administration of the recited compound in rats. *See*, the specification, page 43, Example 6, and Figure 1. The specification also discloses that the recited compound has been shown to inhibit PDE4 *in vitro*. *See*, specification, page 42, line 10 to page 43, line 4, Examples 4 and 5. The IC₅₀ value of the recited compound for PDE4 inhibition was 4.4 μM, as compared to 67 μM and 15 μM of (+) isomer and racemic compound, respectively. *See* Table 1 on page 42. Applicants point out that the examples and data are certainly sufficient to satisfy the enablement requirement under 35 U.S.C. § 112, first paragraph.

To the extent that the IC₅₀ data provided herein are *in vitro*, Applicants point out that to demonstrate utility, Applicants need only show that any given compound is pharmacologically active *in vitro*. *See Cross v. Iizuka*, 753 F.2d 1040, 1051 (Fed. Cir. 1985) (“Successful *in vitro* testing will marshal resources and direct the expenditure of effort to further *in vivo* testing of the most potent compounds, thereby providing an immediate benefit

to the public, analogous to the benefit provided by the showing of an *in vivo* utility.) (citations omitted). Further, “[i]f a statement of utility in the specification contains ... a connotation of how to use, and/or the art recognizes that standard modes of administration are known and contemplated,” the enablement requirement is satisfied. *Manual of Patent Examination and Procedure* § 2164.01(c) (citing, *inter alia*, *In re Brana*, 51 F.3d 1560, 1566 (Fed. Cir. 1993)).

Moreover, “[a]n *in vitro* or *in vivo* animal model in the specification, in effect, constitutes a ‘working example’ if the example ‘correlates’ with a disclosed or claimed method” (MPEP § 2164.02). Explaining further, MPEP § 2164.02 states:

“[I]f the state of the art is such that a particular model is recognized as correlating to a specific condition, then it should be accepted as correlating unless the examiner has evidence that the model does not correlate. Even with such evidence, the examiner must weigh the evidence for and against correlation and decide whether one skilled in the art would accept the model as reasonably correlating to the condition.”

Finally, MPEP § 2164.02 also recognizes that “a rigorous correlation is not necessary where the disclosure of pharmacological activity is reasonable based upon the probative evidence” (quoting *Cross v. Iizuka*, 753 F.2d 1040, 1050, 224 USPQ 739, 747 (Fed. Cir. 1985)).

With this legal framework in mind, Applicants respectfully submit publications to show the correlations between the claimed methods and the *in vitro* model for PDE4 inhibition described in the specification (*e.g.*, Example 4 on page 42, lines 10-24) as Exhibit A (MULLER *et al.*, 1998, “Thalidomide analogs and PDE4 inhibition,” *Bioorg. Med. Chem. Lett.*, 2669-2674, and CORRAL *et al.*, 1999, “Immunomodulation by thalidomide and thalidomide analogues,” *Ann. Rheum. Dis.* 58(Suppl 1):1107-1113). Applicants note that human working examples are not required under 35 U.S.C., first paragraph, as explained in MPEP § 2164.02.

In view of the foregoing, it is clear that a sufficient guidance is provided in the specification so as to allow those of ordinary skill in the art to make and use the claimed invention.

Further, Applicants note that some factors that may --but need not¹-- be considered in determining whether experimentation is undue include the quantity of experimentation necessary and the amount of direction or guidance provided. *In re Wands*, 858 F.2d 731, 737 (Fed. Cir. 1988). In *In re Wands*, the Court of Appeals for the Federal Circuit held that claims directed to immunoassay methods were enabled even though in order to practice the claimed invention, one would have to screen “hybridomas to determine which ones secrete antibody with desired characteristics.” This was because “[p]ractitioners of this art are prepared to screen negative hybridomas in order to find one that makes the desired antibody.” *Id.* at 740.

The claimed invention is directed to the use of obtainable compound, for which routes of administration and amounts are set forth in the specification on pages 22-25. The skilled artisan can readily determine the IC₅₀ for the compound by using the methods described in the specification at page 42. The IC₅₀ value that he/she determines is a good indication that the compound is useful in the treatment of the diseases recited in the claims. Moreover, the determination by a physician as to whether the claimed compound is effective in treating the recited diseases, for example, asthma, in a given patient is a type of determination that is always made by physicians for every pharmaceutical. Indeed, the determination is a routine one that every physician is prepared to make, and which requires little or no effort. Therefore, Applicants respectfully submit that one reasonably skilled in the art could make or use the invention as claimed without undue experimentation.

In sum, Applicants respectfully submit that: (1) the specification provides sufficient information and guidance to those of ordinary skill in the art to make and use the claimed invention; (2) the Examiner did not provide any factual or legal basis to doubt that the claims are enabled; and (3) to the extent any experimentation is necessary, such experimentation is not undue. Therefore, Applicants respectfully request that the rejection of the claims under 35 U.S.C. § 112, first paragraph be reconsidered and withdrawn.

¹ *Amgen, Inc. v. Chugai Pharmaceutical Co., Ltd.*, 927 F.2d 1200, 1230 (Fed. Cir. 1991), *cert. denied*, 502 U.S. 856 (1991) (“it is not necessary that a court review all the *Wands* factors to find a disclosure enabling. They are illustrative, not mandatory.”).

Conclusion

In view of the foregoing, all the rejections of the claims should be withdrawn. Reconsideration, entry of the above amendment and remarks, and allowance of the pending claims are respectfully requested. Should the Examiner not agree that all claims are allowable, a personal or telephonic interview is respectfully requested to discuss any remaining issues and to accelerate the allowance of the above-identified application.

No fee is believed due for this submission. However, if any fees are required for the entry of this paper or to avoid abandonment of this application, please charge the required fees to Jones Day Deposit Account No. 503013.

Respectfully submitted,

Date: May 10, 2006


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LETTERS

THALIDOMIDE ANALOGS AND PDE4 INHIBITION

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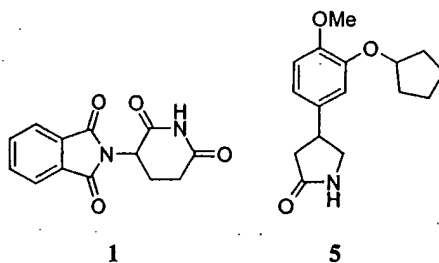
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Abstract. N-Phthaloyl 3-amino-3-arylpropionic acid analogs of thalidomide that are potent inhibitors of tumor necrosis factor- α are reported. These compounds were found to be potent inhibitors of phosphodiesterase 4.

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Introduction

Tumor necrosis factor- α (TNF- α) is a key cytokine in the inflammatory cascade. Excessive TNF- α levels have been found to be associated with a number of inflammatory and autoimmune conditions including rheumatoid arthritis, Crohn's disease, aphthous ulcers, erythema nodosum leprosum in leprosy, septic shock, cachexia, graft versus host disease, asthma, ARDS, and AIDS.¹ Thus, control of TNF- α levels could be a key to the treatment of a wide range of diseases. The validity of this approach has recently been demonstrated by the clinical benefit observed in the treatment of rheumatoid arthritis and Crohn's disease by TNF- α antibodies and TNF- α soluble receptors.² In 1991, thalidomide (1) was reported to be a selective inhibitor of TNF- α production in activated monocytes.³ Although thalidomide has a tragic history because of its teratogenic properties, it has never totally disappeared from pharmaceutical use because of its effective immunomodulatory properties.⁴ In a program to increase the TNF- α inhibitory potency of thalidomide and eliminate/decrease its teratogenic potency we have prepared numerous analogs of thalidomide.



We recently reported on a series of thalidomide analogs (2 and 3) derived from 3-amino-3-arylpropionic acids, which are potent inhibitors of TNF- α .⁵ This series of thalidomide analogs are much more potent inhibitors of TNF- α than thalidomide (TNF- α IC₅₀ = ~200 μ M)⁵. The mechanism of thalidomide's inhibition of TNF- α levels is unknown, although it was reported by the Kaplan group that it decreases TNF- α mRNA stability.⁶ We have continued to explore the mechanism of action of thalidomide and these analogs. It is well documented that elevated levels of cAMP inhibit TNF- α production in activated monocytes and peripheral blood mononuclear

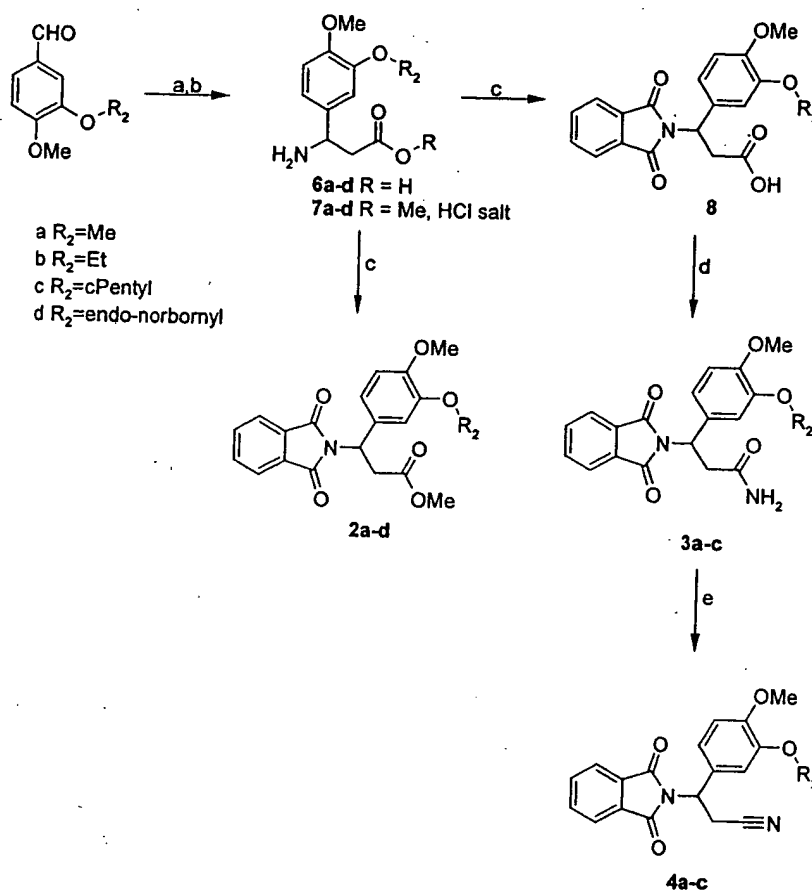
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PII: S0960-894X(98)00475-2

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EXHIBIT A

cells (PBMC).⁷ Cellular levels of cAMP are controlled by adenylate cyclase and the cAMP phosphodiesterases (PDEs).⁸ PDE4 is the major enzyme found in monocytes, the major producers of TNF- α in the inflammatory cascade. Inhibition of PDE4 has been shown to be an effective method for inhibition of TNF- α production in activated monocytes and PBMC. We wish to report the discovery that these thalidomide analogs (2 and 3) and the related nitriles (4) are potent inhibitors of PDE4.

Scheme 1



Regents: (a) NH_4OAc , $\text{CH}_2(\text{CO}_2\text{H})_2$, EtOH, reflux; (b) $\text{SOCl}_2/\text{MeOH}$; (c) N-carboxyphthalimide, Na_2CO_3 , $\text{CH}_3\text{CN}/\text{H}_2\text{O}$; (d) (1) CDI/THF, (2) concentrated NH_4OH ; (e) SOCl_2 or $(\text{COCl})_2/\text{DMF/pyridine}$.

Chemistry

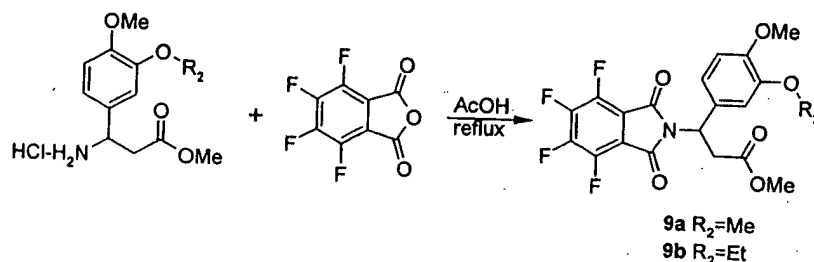
The ester and amide analogs were prepared as previously described (Scheme 1).⁵ The nitrile analogs were prepared from the corresponding amides. 3-Amino-3-arylpropionic acids were prepared as previously described by treatment of a substituted benzaldehyde with malonic acid and ammonium acetate in refluxing EtOH. Substituted 3,4-dialkoxybenzaldehydes were commercially available or prepared as previously described.⁹ The N-phthaloyl carboxylic acids (8a-c) were prepared using a standard Nef reaction. The N-

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phthaloyl carboxylic methyl esters **2a-d** were prepared by conversion of the 3-amino-3-arylpropionic acid (**6a-d**) to the methyl esters (**7a-d**) by treatment with SOCl_2 in MeOH, followed by treatment with Nef's reagent in the presence of Na_2CO_3 (Scheme 1). Conversion of phthaloyl carboxylic acids (**8a-c**) to the corresponding phthaloyl amides (**3a-c**) was accomplished by activation of the carboxylic acid with carbonyldiimidazole (CDI) followed by treatment with conc. NH_4OH . The amides **3a-c** were dehydrated to the nitriles with SOCl_2 or $(\text{COCl})_2$.¹⁰ The tetrafluorophthaloyl analogs **9a** and **9b** were prepared by condensation of **7a** and **7b**, respectively with tetrafluorophthalic anhydride in refluxing acetic acid (Scheme 2).

Scheme 2



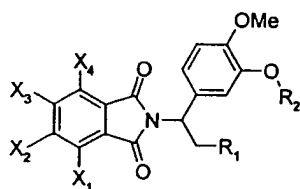
Biological Assays

$\text{TNF-}\alpha$ inhibitory activity was measured in lipopolysaccharide (LPS) stimulated PBMC as previously reported.⁵ Crude PDE4 extract was obtained from U937 cells using the method of Hill and Mitchell as described below.¹¹ Cells (1×10^9) were washed in PBS and lysed in cold homogenization buffer (20 mM Tris-HCl, pH 7.1, 3 mM 2-mercaptoethanol, 1 mM MgCl_2 , 0.1 mM EGTA, 1 μM PMSF, 1 $\mu\text{g/mL}$ leupeptin). Following homogenization in a Dounce homogenizer the supernatant was collected by centrifugation and loaded onto a Sephacryl S-200 column equilibrated in homogenization buffer. PDE was eluted in homogenization buffer and the rolipram sensitive fractions pooled and stored in aliquots. PDE activity was assayed using the protocol adapted from Hill and Mitchell¹¹ and based on assay described by Thompson et al.¹² Enzyme activity was assayed in 50 mM Tris-HCl, pH 7.5, 5 mM MgCl_2 and 1 μM cAMP (of which 1% was ^3H cAMP) in the presence of varying concentrations of inhibitors. The amount of extract used was pre-determined to ensure that reactions were within the linear range and consumed less than 15% of the total substrate. Reactions were performed at 30 °C for 30 min and terminated by boiling for 2 min. The samples were then chilled and treated with snake venom (1 mg/mL) at 30 °C for 15 min. Unused substrate was removed by incubation with 200 μL AG1-X8 resin (BioRad) for 15 min. Samples were then spun at 3000 rpm for 5 min and 50 μL of the aqueous phase taken for counting. Each data point was carried out in duplicate with activity expressed as percentage of control. IC_{50}s was determined from dose response curves derived from three independent experiments. $\text{TNF-}\alpha$ and PDE4 IC_{50}s were calculated by non-linear regression analysis (variable slope) using Prism by GraphPad Software, Inc.

Results and Discussion

These thalidomide analogs were screened for their ability to inhibit TNF- α in LPS stimulated human PBMC.⁵ PDE4 inhibitory activity was assayed with PDE4 enzyme isolated from the U937 cells, a promonocytic cell line. A good correlation between TNF- α inhibition and PDE4 inhibition was observed for the majority of compounds (Table 1). Thalidomide was inactive in the PDE4 assay ($IC_{50} > 500 \mu M$). A separation in mechanism of action between these analogs and thalidomide was found. These compounds appear to inhibit TNF- α by elevation of cellular cAMP levels.

Table 1: TNF- α and PDE4 Inhibition by Thalidomide



compd	R ₂	R ₁	X ₁	X ₂	X ₃	X ₄	TNF- α	PDE4
							IC ₅₀ [μM]	IC ₅₀ [μM]
2a	Me	CO ₂ Me	H	H	H	H	2.9	2.5
2b	Et	CO ₂ Me	H	H	H	H	0.70	0.23
2c	cPentyl	CO ₂ Me	H	H	H	H	1.6	1.7
2d	norbornyl	CO ₂ Me	H	H	H	H	2.4	0.74
3a	Me	CONH ₂	H	H	H	H	13	9.4
3b	Et	CONH ₂	H	H	H	H	2.7	2.0
3c	cPentyl	CONH ₂	H	H	H	H	2.5	1.1
4a	Me	CN	H	H	H	H	1.7	1.3
4b	Et	CN	H	H	H	H	0.12	0.13
4c	cPentyl	CN	H	H	H	H	1.6	0.35
9a	Me	CO ₂ Me	F	F	F	F	0.26	4.7
9b	Et	CO ₂ Me	F	F	F	F	0.38	2.2

The prototypical PDE4 inhibitor, rolipram (PDE4 IC_{50} = 0.40 μM and TNF- α IC_{50} = 0.15 μM),¹³ 5 contains a 3-cyclopentoxy-4-methoxyphenyl moiety, which correlated with the 3,4-dimethoxyphenyl moiety found in these thalidomide analogs. The SAR of the 3,4-dialkoxyphenyl moiety in rolipram type PDE4 inhibitors is well developed. A 3-cyclopentoxy-4-methoxyphenyl moiety along with other large hydrophobic 3-

alkoxy substituents such as endo-norbornyloxy is preferred.¹⁴ The SAR of the 3-alkoxy group was explored in this series (Table 1). Interestingly, these PDE4 inhibitors do not directly follow the SAR of known rolipram type analogs. The 3-cyclopentoxo-4-methoxyphenyl analog **2c** was only 2-fold more active as a PDE4 inhibitor than the previously reported 3,4-dimethoxy analog **2a**. The 3-ethoxy-4-methoxy analog **2b** was over 7-fold more active as a PDE4 inhibitor than **2c**. The differences in TNF- α inhibitory activities were smaller but followed the same trend. The smaller differences in the TNF- α inhibitory potencies are probably due to cell based effects of the TNF- α inhibition in which the compound must enter the cell to be active. In the amide series, the 3-cyclopentoxyl analog **3c** was 2-fold more active than the 3-ethoxy analog **3b** and a magnitude more active as a PDE4 inhibitor. However, **3b** and **3c** were equipotent as TNF- α inhibitors. Isosteric replacement of the amide/ester moiety with a nitrile was investigated. The nitriles **4a-c** were significantly more potent as PDE4 inhibitors than the amide analogs but afforded only slight improvements over the ester analogs. The 3-ethoxy-4-methoxy nitrile is the most potent TNF- α inhibitor of the reported compounds with an IC_{50} of 120 nM. In this series of compounds the smaller 3-ethoxy substituent appears to be preferred over larger 3-alkoxy substituent.

Recently other researchers reported the tetrafluorophthaloyl analog of **3a** as a potent TNF- α inhibitor in LPS stimulated THP-1 cells.¹⁵ We also prepared this compound, **9a** and the 3-ethoxy-4-methoxy analog **9b** and found them to be potent inhibitors of TNF- α in LPS stimulated PBMC (Table 1). However, both compounds were found to be cytotoxic at 10 and 100 μ M in this assay which put the TNF- α inhibition results in question.¹⁶ The non-fluorinated analogs described were not cytotoxic at the highest concentration (100 μ M) tested in this assay. Both **9a** and **9b** were found to be approximately 10- to 15-fold less active as PDE4 inhibitors compared to their TNF- α inhibitory activity. Whether the differences in the TNF- α and PDE4 inhibitory activity are due to the cytotoxicity of the compounds in the TNF- α inhibition is unknown.

In conclusion, we have determined that these thalidomide analogs are potent inhibitors of PDE4. It is proposed that these thalidomide analogs control TNF- α levels by inhibition of PDE4. Thalidomide was found to be inactive against PDE4 ($IC_{50} > 500 \mu$ M). Although thalidomide was inactive against PDE4 the possibility that one or more of its metabolites or degradation products inhibits PDE4 has not been eliminated. Using thalidomide as a lead structure we have discovered a novel series of potent PDE4 inhibitors. The most active compound reported here is >1,500 times more potent (IC_{50}) as a TNF- α inhibitor than thalidomide. Future publications from our laboratories will further describe the SAR and report the potential therapeutic value of these PDE4 inhibitors.

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16. Cytotoxicity was determined as described in reference 5.


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Immunomodulation by thalidomide and thalidomide analogues

Laura G Corral, Gilla Kaplan

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Tumour necrosis factor α (TNF α), a key cytokine involved in the host immune response, also contributes to the pathogenesis of both infectious and autoimmune diseases. To ameliorate the pathology resulting from TNF α in these clinical settings, strategies for the inhibition of this cytokine have been developed. Our previous work has shown that the drug thalidomide is a partial inhibitor of TNF α production in vivo. For example, when leprosy patients suffering from erythema nodosum leprosum (ENL) are treated with thalidomide, the increased serum TNF α concentrations characteristic of this syndrome are reduced, with a concomitant improvement in clinical symptoms. Similarly, we have found that in patients with tuberculosis, with or without HIV infection, short-term thalidomide treatment reduces plasma TNF α levels in association with an accelerated weight gain. In vitro, we have also shown that thalidomide partially inhibits TNF α produced by human peripheral blood mononuclear cells (PBMC) responding to stimulation with lipopolysaccharide (LPS). Recently, we found that thalidomide can also act as a costimulatory signal for T cell activation in vitro resulting in increased production of interleukin 2 (IL2) and interferon γ (IFN γ). We also observed a bidirectional effect on IL12 production: IL12 production is inhibited by thalidomide when PBMC are stimulated with LPS, however, IL12 production is increased in the presence of the drug when cells are stimulated via the T cell receptor. The latter effect is associated with upregulation of T cell CD40 ligand (CD40L) expression. Thus, in addition to its monocyte inhibitory activity, thalidomide exerts a costimulatory or adjuvant effect on T cell responses. This combination of effects may contribute to the immunomodulating properties of the drug.

To obtain drugs with increased anti-TNF α activity that have reduced or absent toxicities, novel TNF α inhibitors were designed using thalidomide as template. These thalidomide analogues were found to be up to 50 000 times more active than thalidomide. The compounds comprise two different types of TNF α inhibitors. One class of compounds, shown to be potent phosphodiesterase 4 (PDE4) inhibitors, are selective TNF α inhibitors in LPS stimulated PBMC and have either no effect or a suppressive effect on T cell activation. The other class of compounds also inhibit TNF α production, but do not inhibit PDE4 enzyme. These compounds are also potent inhibitors of several LPS induced monocyte inflammatory cytokines. Also, the latter compounds markedly

stimulate the anti-inflammatory cytokine IL10. Similarly to thalidomide, these drugs that do not inhibit PDE4 act as costimulators of T cells but are much more potent than the parent drug. The distinct immunomodulatory activity of these new TNF α inhibitors may potentially allow them to be used in the clinic for the treatment of a wide variety of immunopathological disorders of different aetiologies.

TNF α is a key player in the immune response

TNF α is a pleiotropic cytokine produced primarily by monocytes and macrophages, but also by lymphocytes and NK cells. TNF α plays a central part in the host immune response to viral, parasitic, fungal and bacterial infections. The importance of TNF α and TNF α signalling through its receptors in the host immune response to disease has become clearer as a result of a number of seminal studies. For example, mice genetically deficient in TNF α have a significantly reduced humoral immune response to adenovirus infection.¹ In *Leishmania major* infection, TNF α signalling is important for protection as mice lacking TNF α p55 receptor (TNFR-p55) show delayed elimination of the parasites compared with controls and the lesions formed failed to resolve.² Mice deficient in TNFR-p55 are also significantly impaired in their ability to clear infection with *Candida albicans* and readily succumb to the infection. TNF α signalling is also crucial in resisting *Streptococcus pneumoniae* infections in mice.³ In addition, TNF α is essential for protection against murine tuberculosis. TNFR-p55 deficient mice have been shown to be more susceptible to tuberculosis infection. When TNF α was neutralised in vivo by monoclonal antibodies impaired protection against mycobacterial infection was observed.^{4,5} The data from both models also established that TNF α and the TNFR-p55 are essential for production of reactive nitrogen intermediates by macrophages early in infection.

TNF α contributes to disease pathogenesis
Although TNF α is crucial to the protective immune response, it also plays a part in the pathogenesis of both infectious and autoimmune diseases. Increased concentrations of TNF α have been shown to trigger the lethal effects of septic shock syndrome.⁶ TNF α has also been implicated in the development of cachexia, the state of malnutrition that complicates the course of chronic infections and many cancers.⁷ In rheumatoid arthritis, TNF α is a critical mediator of joint inflammation and therefore an important therapeutic target.

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Recently, it has been shown that treatment of patients with neutralising anti-TNF α antibodies produces a dramatic reduction in disease activity in this condition.⁸ Similarly, it has been shown that in inflammatory bowel disease, neutralisation of TNF α results in a profound amelioration of clinical symptoms.^{9,10} Reductions in TNF α levels have also been linked with a significant reduction of clinical symptoms in leprosy patients with ENL, including fever, malaise, and arthritic and neuritic pain.¹¹ In tuberculosis patients, reduction of TNF α levels was associated with accelerated weight gain.¹²

Thalidomide inhibits TNF α production by monocytes

The pathology associated with TNF α production is profound and in many diseases leads to significant morbidity and mortality. This has led to a concerted effort to discover drugs that will down regulate the production of this cytokine. Agents conventionally used in these diseases may inhibit TNF α production, but are also often broadly immunosuppressive (for example, cyclosporin A and corticosteroids) and therefore associated with extensive side effects.¹³ Drugs that are potentially more specific in inhibiting TNF α are under active investigation and development. Our previous work has shown that the drug thalidomide (α -N-phthalimidoglutarimide) is a relatively selective inhibitor of TNF α production by human monocytes *in vivo*. This property of thalidomide was first described in leprosy patients with ENL, an acute inflammatory complication of lepromatous leprosy that is accompanied by increased serum TNF α levels. Thalidomide treatment of patients with ENL was shown to induce a prompt reduction of TNF α serum levels with a concomitant abrogation of clinical symptoms.¹¹ Furthermore, in patients with tuberculosis, with or without concomitant HIV infection, thalidomide treatment was found to both decrease plasma TNF α protein levels as well as monocyte TNF α mRNA levels. This decrease was associated with an accelerated weight gain.¹² In a rabbit model of mycobacterial meningitis, thalidomide treatment combined with antibiotics produced a marked reduction in TNF α levels, leucocytosis, and brain disease.¹⁴ In addition, thalidomide inhibited TNF α serum levels in mice challenged with LPS thus partially protecting the animals from septic shock.¹⁵

In vitro, we have found that thalidomide selectively reduces the production of TNF α by human monocytes cultured in the presence of both LPS and mycobacterial products.¹⁶ However, this inhibition was only partial (50% to 70%) possibly because of the instability of the drug in aqueous solutions.¹⁷ The mechanism by which thalidomide reduces TNF α production is still unclear. The drug seems to inhibit TNF α production by human monocytes *in vitro* in association with enhanced degradation of TNF α mRNA.¹⁸ It also inhibits the activation of the nuclear factor κ B (Nf κ B),^{19,20} a promoter for the transcription of TNF α as well as transcription of HIV-1.^{21,22}

Thalidomide has T cell costimulatory properties

Recently, we reported that thalidomide also has a hitherto unappreciated immunomodulatory effect: the drug was shown to costimulate human T cells *in vitro*, synergising with stimulation via the T cell receptor complex to increase IL2 mediated T cell proliferation and T cell IFN γ production.²³ Optimal T cell activation requires two signals.²⁴ The first signal or signal 1 is delivered by clustering of the T cell antigen-receptor-CD3 complex through engagement of specific foreign peptides bound to MHC molecules on the surface of an antigen presenting cell (APC). Signal 1 can be mimicked by crosslinking the T cell receptor (TCR) complexes with anti-CD3 antibodies. Signal 2 (or costimulation) is antigen independent and may be provided by cytokines or by surface ligands on the APC that interact with their receptors on the T cell. Costimulatory signals are essential to induce maximal T cell proliferation and secretion of cytokines, including IL2, which ultimately drive T cell clonal expansion. As antigenic stimulation in the absence of costimulatory signals leads to T cell anergy or apoptosis, costimulation is critically important in the induction and regulation of cellular immunity.

Thalidomide appears to act as a costimulator to T cells that have received signal 1 via the TCR.²³ In our experiments *in vitro*, stimulation of purified T cells with anti-CD3 antibodies, in the absence of signal 2, induced only minimal T cell proliferation. However, the addition of thalidomide to this cell culture system resulted in a concentration dependent increase in proliferative responses.^{23,25} The thalidomide mediated costimulation of T cell proliferation was accompanied by increases in IL2 and IFN γ production. It is noteworthy that in the absence of anti-CD3, there was no T cell proliferative response to thalidomide, indicating that the drug is not mitogenic in itself. It is also interesting to note that in these experiments, thalidomide did not inhibit TNF α production by purified T cells stimulated by anti-CD3 antibodies. This is in contrast with the effects of the drug on TNF α produced by monocytes. As already described above, thalidomide inhibits monocyte TNF α production. The costimulatory effect of thalidomide was greater on the CD8 $^{+}$ T cells than on the CD4 $^{+}$ T cell subset.²³

In addition to its effects on T cell proliferation and T cell cytokine production, we observed that thalidomide induced the up-regulation of CD40L expression on activated T cells.^{23,26} CD40L/CD40 interaction occurs early in the sequence of signalling events between T cells and antigen presenting cells (APC). Signalling through CD40 has been shown to activate APC and to induce expression of costimulatory molecules such as B7, as well as stimulating production of IL12.^{27,28} Thus, CD40 signalling results in a stimulatory feedback mechanism in which the activated APC amplifies the T cell response.²⁹ It has also been suggested that CD40L function is essen-

tial for the survival of CD8+ T cells and that in its absence these cells die or become anergic.³⁰

These studies show that in addition to its inhibitory effect on the production of monocyte cytokines, thalidomide exerts a costimulatory or adjuvant effect on T cell responses. The immune modulating effects of the drug in patients may thus be attributable to a balance between the inhibition of production of monocyte cytokines, including TNF α , and the costimulation of T cell activity. The effects of thalidomide in vivo in HIV infected patients seem to reflect the costimulatory activity of the drug.²⁸ In a placebo controlled study to evaluate the effects of in vivo immunomodulation with thalidomide, the drug was administered for four weeks to HIV infected patients. Thalidomide treatment did not affect TNF α levels in these patients. In contrast, thalidomide treatment resulted in significant immune stimulation. This was reflected by increases in DTH responses and increased plasma levels of T cell activation markers such as soluble IL2 receptor (sIL2R) and soluble CD8 antigen. An earlier study of tuberculosis patients treated with thalidomide showed increased plasma levels of IFN γ suggesting an immunostimulatory effect of the drug.¹² Recently, patients suffering from sarcoidosis have shown consistent increases in sIL2R plasma levels after thalidomide treatment (Oliver *et al*, manuscript in preparation). In the same study, thalidomide treatment increased the proliferation of sarcoid patient T cells in response to concanavalin A in vitro. These results strongly suggest that thalidomide directly stimulates T cells in vivo in patients, corresponding to the T cell costimulatory properties of the drug observed in vitro in T cells from normal donors,^{22, 23} as well as in the T cells of HIV infected patients.²⁸

Thalidomide analogues are improved TNF α inhibitors

In addition to being the drug of choice for the treatment of ENL, thalidomide has been shown to be useful in a number of clinical situations including rheumatoid arthritis, HIV associated aphthous ulcers and chronic graft versus host disease.³¹⁻³⁴ However, thalidomide is a potent teratogen and ingestion of the drug by a pregnant woman can lead to catastrophic

birth defects.³⁵ In addition, thalidomide treatment is often accompanied by a number of side effects, including peripheral neuropathy.³⁶ Therefore, the use of thalidomide requires strict monitoring of all patients.³⁷ Thus, there is a pressing need to develop drugs with increased TNF α inhibitory activity and reduced or absent toxicities. Towards this end, structural analogues of thalidomide have been designed and synthesised at Celgene Corporation (Warren, New Jersey) and screened for inhibition of TNF α production. A large number of potent novel TNF α inhibitors were thus identified. Recently, some of these compounds were described.^{20, 38-40} On a molar basis, the more potent of these thalidomide analogues were found to be up to 50 000-fold more potent than thalidomide at inhibiting TNF α production by human PBMC stimulated by LPS in vitro. Furthermore, we have shown that some of these compounds retain high activity in LPS stimulated human whole blood.⁴⁰ In vivo, several of these new compounds showed improved activity in reducing LPS induced TNF α levels in mice¹⁷ and in inhibiting the development of adjuvant arthritis in rats.^{40a}

Thalidomide analogues comprise two distinct classes of molecules

A group of thalidomide analogues, selected for their capacity to potentially inhibit TNF α production by LPS stimulated PBMC, was further investigated (fig 1). When tested for their effect in vitro on LPS induced cytokines, different patterns of cytokine modulation were shown.²³ One class of compounds, class I or ImiDs (Immunomodulatory Imide Drugs) showed not only potent inhibition of TNF α but also marked inhibition of LPS induced monocyte IL1 β and IL12 production. LPS induced IL6 was also inhibited by these drugs, albeit partially. These drugs were potent stimulators of LPS induced IL10, increasing IL10 levels by 200-300%. In contrast, the other class of compounds, class II or SelCiDs (Selective Cytokine Inhibitory Drugs), while still potentially inhibiting TNF α production, had a more modest inhibitory effect on LPS induced IL1 β and IL12, and did not inhibit IL6 even at high drug concentrations. In addition, SelCiDs produced a more modest IL10 stimulation (20-50% increases). In all of these characteristics, SelCiDs were more similar to thalidomide than ImiDs.^{16, 17}

Further characterisation of the SelCiDs showed that they are potent PDE4 inhibitors.³⁹ PDE4 is one of the major phosphodiesterase isoenzymes found in human myeloid and lymphoid lineage cells.⁴¹ The enzyme plays a crucial part in regulating cellular activity by degrading the ubiquitous second messenger cAMP and maintaining it at low intracellular levels. Inhibition of PDE4 results in increased cAMP levels leading to the modulation of LPS induced cytokines including inhibition of TNF α .⁴² Increasing intracellular cAMP levels have been shown to inhibit TNF α production in monocytes as well as in lymphocytes,^{41, 43} although it is not clear how this inhibition is

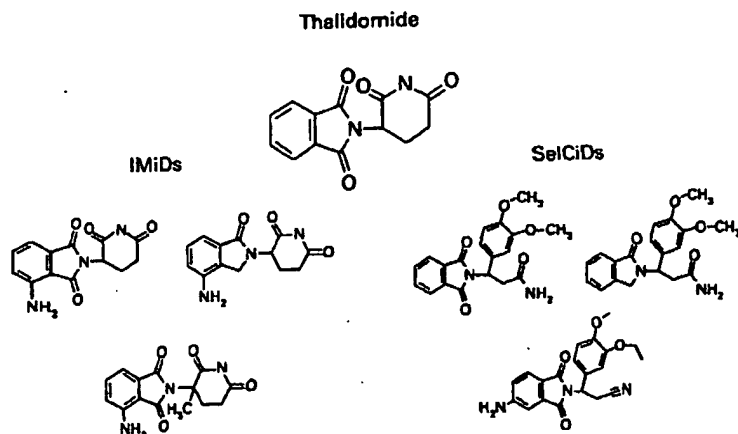


Figure 1 Chemical structures of thalidomide and selected thalidomide analogues.

regulated. Interestingly, the IMiDs and thalidomide were found not to inhibit PDE4.⁴⁰

In addition to the differential modulation of LPS induced monocyte cytokines, the two classes of compounds showed distinct effects on T cell activation. SelCiDs, the PDE4 inhibitors, had little effect on T cell activation causing only a slight inhibition of T cell proliferation. This effect was not unexpected as it is well established that increasing cAMP levels in T cells during the early phase of mitogen or antigen activation results in a decrease in proliferative potential.⁴⁴ On the other hand, IMiDs, the non-PDE4 inhibitors, were potent costimulators of T cells and increased cell proliferation dramatically in a dose dependent manner.³³ Similarly to thalidomide, these compounds had a greater costimulatory effect on the CD8+ T cell subset than on the CD4+ T cell subset (Corral *et al*, unpublished observation). IMiDs, when added to anti-CD3 stimulated T cells, also caused marked increases in the secretion of IL2 and IFN γ and induced the up-regulation of CD40L expression on T cells.³³ These findings show that in addition to their strong anti-inflammatory properties, IMiDs efficiently costimulate T cells with 100 to 1000 times the potency of the parent drug. The molecular target of these co-stimulatory cytokine modulating drugs is as yet unknown.

Thalidomide and IMiDs modulate cytokines differently according to cell type and stimulation pathway

As described above, thalidomide has been shown to inhibit IL12 production by LPS stimulated monocytes *in vitro*.^{33,45} *In vivo*, however, thalidomide treatment of HIV infected²⁶ and *M tuberculosis* infected patients induced increases in plasma IL12 levels (Bekker *et al*, submitted data). Thalidomide treatment also resulted in increases in plasma IL12 levels in patients with scleroderma and sarcoidosis (Oliver *et al*, manuscripts in preparation). These dual and opposite effects of thalidomide may be explained by the differential modulation of cytokines according to target cell type and specific pathways of cellular stimulation.

IL12 is produced primarily by APC (monocytes/macrophages and dendritic cells) and is regulated by both T cell dependent and T cell independent pathways. LPS directly induces T cell independent IL12 production by APC, which is inhibited by thalidomide. In the T cell dependent pathway, on the other hand, the production of IL12 by the APC is

induced primarily by the interaction of CD40 on the surface of the APC with CD40L on the surface of activated T cells.^{34,46} When T cells were stimulated by anti-CD3, thalidomide and IMiDs treatment caused a significant stimulation of IL12 production.³³ Thalidomide and IMiDs also induced an up-regulation of CD40L on the surface of T cells.^{33,39} Blockade of this pathway inhibits the production of IL12 and abolishes the stimulatory effect of thalidomide.³⁶ Interestingly, in HIV infected patients, the consistent increases in plasma IL12 levels induced by thalidomide treatment lagged behind the increases in T cell activation markers.³⁶ This observation suggested that IL12 production was augmented as a consequence of drug induced T cell activation.

The dichotomous nature of thalidomide cytokine modulation may explain the seemingly opposite effects observed in different clinical situations. When patients with Behçer's syndrome are treated with thalidomide, healing of inflammatory aphthous ulcers occurs, but is sometimes accompanied by exacerbation of erythema nodosum.⁴⁷ Similarly, the paradoxical worsening of graft versus host disease⁴⁸ and toxic epidermal necrolysis⁴⁹ reported in clinical trials of thalidomide may be a manifestation of the unsuspected immune stimulatory effect of this drug.

Potential clinical applications of thalidomide and thalidomide analogues

The thalidomide analogues discussed here seem to have retained different properties of the parent drug (table 1). The distinct immunomodulatory activities of these two classes of drugs suggest they may have applications in different immunopathological disorders. SelCiDs, which inhibit PDE4, may be used in clinical situations in which PDE4 inhibition and selective TNF α inhibition are beneficial. Therapeutic increase of intracellular cAMP levels by PDE4 inhibitors has anti-inflammatory effects, which may afford consequent benefits in a variety of diseases such as asthma,⁵⁰ atopic dermatitis⁵¹ and rheumatoid arthritis.⁵² Indeed, in an animal model of adjuvant arthritis, thalidomide derived PDE4 inhibitors have shown efficacy in suppressing the development of disease as measured by ankle swelling, hind limb radiographic changes and weight gain.^{40a} The suppression of arthritis was accompanied by a reduction in TNF α and IL2 mRNA levels in the ankle joints of treated rats.

Table 1 Immunomodulatory profiles of thalidomide and thalidomide analogues

Thalidomide	IMiDs	SelCiDs
Inhibits LPS induced inflammatory cytokines TNF α and IL12	Strongly inhibit LPS induced inflammatory cytokines: TNF α , IL1 β , IL6 and IL12	Strongly inhibit LPS induced inflammatory cytokines TNF α and IL12
Stimulates LPS induced anti-inflammatory cytokine IL10	Strongly stimulate LPS induced anti-inflammatory cytokine IL10	Stimulate LPS induced anti-inflammatory cytokine IL10
Costimulates T cell activation	Strongly costimulate T cell activation	Inhibit or have no effect on T cell activation
Does not inhibit PDE4	Do not inhibit PDE4	Strongly inhibit PDE4

Other known selective PDE4 inhibitors, such as rolipram, have been reported to have dose limiting side effects, such as nausea and vomiting, which limit the therapeutic use of these drugs.^{33,34} These side effects may be produced by the lack of specificity of these drugs—that is, the compounds inhibit one or more PDE isoenzymes in non-target tissues. For example, it is probable that the emetic activity of PDE4 inhibitors is attributable to an action of the drugs in the CNS.³⁵ Intensive effort is being directed towards identifying compounds with improved therapeutic ratios. Preliminary results with thalidomide derived PDE inhibitors indicate that these novel drugs are selective inhibitors of PDE4 and may be better tolerated than other PDE4 inhibitors, as they have not shown evidence of emesis in animals. One of these drugs has been recently shown to be well tolerated in a small human safety trial in the United Kingdom (D Stirling, personal communication).

The IMiDs, as thalidomide, are anti-inflammatory drugs that do not target PDE4. These compounds, in addition to their potential use to decrease inflammation, could also be useful in clinical settings where there is a defect in T cell function, as in HIV disease. HIV infection is accompanied by deficiencies in the production of IL12 and in the up-regulation of CD40L.^{36,37} IL12 has been shown to restore HIV specific cell mediated immunity *in vitro*³⁸ and to increase HIV specific CTL responses *in vitro*³⁹ and *in vivo*.⁴⁰ Also, deficient IL12 responses in HIV infected patients can be restored *in vitro* by CD40L and IFN γ ,⁴¹ the same costimulatory factors induced by thalidomide and IMiDs. Thus, these drugs may eventually be used to restore or stimulate IL12 production in immune deficient patients.

IL12 has also been shown to exhibit potent anti-tumour activity in murine tumour models through various mechanisms including the stimulation of natural killer cell activity,⁴² activation of CD8+ cytotoxic T cells⁴³ and increased IFN γ mediated anti-angiogenesis.⁴⁴ Thalidomide has also recently been reported to exhibit anti-tumour activity through the inhibition of angiogenesis *in vivo*.^{45,46} However, this anti-angiogenic effect does not seem to be mediated by TNF α inhibition. Although these studies did not determine the mechanism of thalidomide's anti-angiogenic activity, it is conceivable that stimulation of IFN γ /IL12 levels may be at least partly responsible. One report indicates that thalidomide may have anti-angiogenic activity in multiple myeloma in humans.⁴⁷

In summary, our recent findings that thalidomide and IMiDs preferentially costimulate CD8+ T cells and induce T cell dependent IL12 production suggest possible applications of these drugs in the control of viral infections^{70,71} or in boosting anti-tumour immunity.^{72,73} Also, there are anecdotal reports of the efficacy of thalidomide in treating refractory inflammatory bowel disease.⁷⁴⁻⁷⁶ Recently, preliminary findings were announced from a pilot study with patients with Crohn's disease refractory to standard treatments (Annual Digestive Disease Meeting, May 1999, Orlando, FL).

In this study, two third of the patients experienced a significant improvement in their condition. This therapeutic effect may be a combination of TNF α inhibition and CD8+ T cell stimulation.^{77,78}

Conclusions

In several disease conditions such as septic shock, chronic infections and cancer, overproduction of TNF α is accompanied by severe toxicities. Thalidomide inhibits TNF α production in different diseases without causing the immunosuppression often associated with standard agents such as glucocorticoids and cyclosporin A. Our results indicate that the immunomodulating effects of thalidomide may occur via the inhibition of TNF α production and/or the stimulation of T cell responses, without the suppression of host immunity.

Recent efforts have concentrated on developing TNF α inhibitors that are efficient, safe and specific. The collaboration between Rockefeller University and Celgene Corporation scientists has led to the discovery of two different classes of immunomodulators derived from thalidomide and selected for their potent anti-TNF α inhibitory activity. Preliminary results indicate that at least some of these new compounds are non-toxic and non-teratogenic.²⁰ The two classes of thalidomide analogues, however, possess distinct properties. IMiDs are potent inhibitors of monocyte inflammatory cytokine production and also are strong costimulators of T cell activity. SelCiDs, on the other hand, are potent PDE4 inhibitors and thus, more selective inhibitors of TNF α . Unlike IMiDs, these compounds do not costimulate T cells but inhibit T cell activity. Thus, the two classes of compounds may prove to be useful in different clinical settings according to their immunomodulatory properties. The thalidomide analogues are being used as investigational tools in animal disease models to define mechanisms of pathogenesis and to continue to elucidate the mechanisms of drug action.

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